

only a few pedigrees. However, there have been reports of specific mutations among various ethnic groups, including p.G717V in blacks (Adjalla et al. 1998), p.N219Y in Caucasians (Acquaviva et al. 2001), and p.R108C in Hispanics (Worgan et al. 2006). Ogasawara et al. (1994b) reported a relatively high incidence of p.E117X in Japanese patients and, more recently, Kobayashi et al. (2006) identified the plural occurrence of each of six mutations (p.L494X, p.R93H, p.E117X, p.R369H, p.G648D, and c.385 + 5G > A) in another Japanese population.

In study reported here, we performed mutation and haplotype analyses on 29 Japanese patients with *mut* MMA to examine the spectrum of mutations within the population and explore the possibility of a molecular diagnosis.

Methods

Patients

Twenty-nine apparently unrelated *mut* MMA patients were studied in the present investigation. There were no consanguineous marriages among the parents of these patients. Patient 6 was a Brazilian descended from Japan immigrants. All of the patients with available clinical information had been symptomatic during their neonatal or infantile periods. Responsiveness to vitamin B12 was not found in all of the patients except patient 7. The oral administration of vitamin B12 in patient 7 reduced the urinary excretion of methylmalonic acid clinically, and the fibroblasts from this patient showed an increased incorporation of ¹⁴C-propionate – from 7 to 40%

– following the administration of vitamin B12 in vitro (case 2 in Kakinuma et al. 1985). This patient was classified as *cbl* MMA on the basis of these findings, but neither the *MMAA* nor *MMAB* mutation was found (Yang et al. 2004). Patient 23 exhibited one of the mutations (p.V669E), but another mutation was not found in the previous study (cell No. 69 in Mikami et al. 1999). The diagnoses of MMA were confirmed by urinary organic acid analysis using gas chromatography and mass spectrometry. The MCM assay was performed in most cases by measuring the isomerization of L-methylmalonyl-CoA to succinyl-CoA by means of high-performance liquid chromatography (Kikuchi et al. 1989), and MCM activity was undetectable in all of those assayed. We could not successfully subclass the patients into *mut*⁰ or *mut*⁻ by this method.

Direct sequencing of the *MUT* genes

Genomic DNA was extracted from cultured fibroblasts, EBV-transformed lymphoblasts, or leukocytes with the aid of a Sepa Gene Kit (Sanko Junyaku, Tokyo, Japan). All coding exons, including flanking introns in *MUT*, were amplified by PCR (Table 1). To facilitate the cycle sequencing analysis, the KS primer sequence and M13 reverse primer sequence were attached to the 5' ends of the sense primers and antisense primers, respectively. The PCR products were directly sequenced using a Big Dye Primer Cycle Sequencing kit and an ABI 310 Genetic Analyzer (PE Applied Biosystems, Foster City, Calif.).

The Ethics Committee of the Tohoku University School of Medicine approved this study.

Table 1 Primers for the amplification of the *MUT* gene

Sense primer ^a		Antisense primer ^b	
MUT-Ex2-KS	KS-GAGTAGCTCCTATTGCCAC	MUT-Ex2-Rev	Rev-GAGTGAATATCATCTTTACA
MUT-Ex3-KS	KS-ACCTTGATTCCAGACTCTTG	MUT-Ex3-Rev	Rev-CTACATTCAAGGAACATATAG
MUT-Ex4-KS	KS-AGTCTGATGATGGTTCATG	MUT-Ex4-Rev	Rev-ATCTAAATCTAGCCTGACAT
MUT-Ex5-KS	KS-TGTACGTGCACTGATCTTAA	MUT-Ex5-Rev	Rev-CTTGTGCCACATTGCTCAGA
MUT-Ex6-KS	KS-GCTATTCTGAAGCTTAATAT	MUT-Ex6-Rev	Rev-TATAAATCTTGACTGTGAAG
MUT-Ex7-KS	KS-TGATGTTTATTTAATTCTGT	MUT-Ex7-Rev	Rev-GTGCATCCATGTATGTGAAA
MUT-Ex8-KS	KS-CTCAGATTGGGATTTGCTGA	MUT-Ex8-Rev	Rev-CACCTCATGCTGTTGTAAGG
MUT-Ex9-KS	KS-ATGCTATGCATCAGGGTCTA	MUT-Ex9-Rev	Rev-ACATGGTTTACAGGATCAAC
MUT-Ex10-KS	KS-GAATTGGATGCATAAAGGCA	MUT-Ex10-Rev	Rev-TTTCTCAGTTGTATGTAAGG
MUT-Ex11-KS	KS-CTTGAAAGATTGCTGTGAA	MUT-Ex11-Rev	Rev-TACCAGTTACCAGGAGATGT
MUT-Ex12-KS	KS-GCCATTAGTATGTTCTGAA	MUT-Ex12-Rev	Rev-ACACTGTCCACTTTTAGACC
MUT-Ex13-KS	KS-TGCCAGTAGTATACCAGTTG	MUT-Ex13-Rev	Rev-GAAGACATAGCTTTACTCTC

^a KS, CGAGGTCGACGGTATCG

^b Rev, CAGGAAACAGCTATGAC

Table 2 Mutations and single nucleotide polymorphisms in the *MUT* gene

Patient number	Gender	Onset	Allele		Single nucleotide polymorphisms											
			Allele 1	Allele 2	c.636	c.1084	c.1495	c.1560	c.1676	c.1677	c.1677	c.1677	c.1808	c.2011		
Pt1	F	7 days	c.322C > T (p.R108C)	c.2098_2099 delAT (p.M700 V fsX10)	G A	A A	G G	ND	A G	A A	ND	A G	A G	C G	C G	A G
Pt2	M	5 days	c.349G > T (p.E117X)	c.349G > T (p.E117X)	G G	G G	G G	16 16	G G	A A	A A	A A	A A	C C	C C	A A
Pt3	M	- ^a	c.349G > T (p.E117X)	c.1481T > A (p.L494X)	G G	G G	G G	16 16	G G	A A	A A	A A	A A	C C	C C	A A
Pt4	M	Neonate	c.349G > T (p.E117X)	c.2179C > T (p.R727X)	G A	G A	G G	16 17	G A	A A	A T	A G	A G	C G	C G	A G
Pt5	M	28 days	c.385+5G > A (IVS2+5G > A)	c.385+5G > A (IVS2+5G > A)	G G	A A	G G	ND	G G	A A	A A	A A	A A	C C	C C	A A
Pt6	M	4 days	c.385+5G > A (IVS2+5G > A)	c.385+5G > A (IVS2+5G > A)	G G	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	A A
Pt7	F	8 months	c.385+5G > A (IVS2+5G > A)	c.1106G > A (p.R369H)	G G	A A	G G	ND	G G	A A	ND	A A	A A	C C	C C	A A
Pt8	M	23 days	c.385+5G > A (IVS2+5G > A)	c.1106G > A (p.R369H)	G A	A A	G G	16 17	G A	A A	A T	A G	A G	C G	C G	A G
Pt9	M	3 days	c.385+5G > A (IVS2+5G > A)	c.1106G > A (p.R369H)	G A	A A	G G	16 17	G A	A A	A T	A G	A G	C G	C G	A G
Pt10	F	- ^a	c.385+5G > A (IVS2+5G > A)	c.1481T > A (p.L494X)	G G	A G	G G	ND	G G	A A	A A	A A	A A	C C	C C	A A
Pt11	M	2 days	c.385+5G > A (IVS2+5G > A)	c.1560G > C (p.K520K, splice error?)	G A	A A	G G	16 17	G A	A A	A T	A G	A G	C G	C G	A G
Pt12	M	1 day	c.753_753 +5del6bp (splice error?)	c.753_753 +5del6bp (splice error?)	G G	A A	G G	16 16	G G	A A	A A	A A	A A	C C	C C	A A
Pt13	F	9 days	c.323G > A (p.R108H)	c.1105C > T (p.R369C)	G A	A A	G G	16 16	G G	A C	A A	A A	A A	C C	C C	A G
Pt14	F	11 months	c.1106G > A (p.R369H)	c.1A > G (p.M1V)	G A	A A	G G	16 16	G G	A C	A A	A A	A A	C C	C C	A G
Pt15	F	10 months	c.1106G > A (p.R369H)	c.1105C > T (p.R369C)	A A	A A	G A	17 16	A G	A C	T A	G A	G A	G C	G C	G G

Table 2 continued

Patient number	Gender	Onset	Allele 1	Allele 2	Single nucleotide polymorphisms										
					c.636	c.1084	c.1495	c.1560	c.1595	c.1676	c.1677	c.1677	c.1677	c.1808	c.2011
Pt16	F	- ^a	c.1106G > A (p.R369H)	Nil found	A G	A A	G G	17 16	A G	A A	T A	G A	G C	G C	G A
Pt17	M	- ^a	c.1481T > A (p.L494X)	c.1481T > A (p.L494X)	G G	G G	G G	16 16	G G	A A	A A	A A	C C	C C	A A
Pt18	M	- ^a	c.1481T > A (p.L494X)	c.1481T > A (p.L494X)	G G	G G	G G	16 16	G G	A A	A A	A A	C C	C C	A A
Pt19	M	- ^a	c.1481T > A (p.L494X)	c.1105C > T (p.R369C)	G A	G A	G G	16 16	G G	A C	A A	A A	C C	C C	A G
Pt20	M	14 days	c.1481T > A (p.L494X)	c.1560+1G > T (IVS8+1G > T)	G A	G A	G G	16 17	G A	A A	A T	A G	C G	C G	A G
Pt21	F	3 days	c.1481T > A (p.L494X)	Nil found	G A	G A	G G	16 17	G A	A A	A T	A G	G C	G C	A G
Pt22	M	- ^a	c.1481T > A (p.L494X)	Nil found	G A	G A	G G	16 16	G G	A C	A A	A A	C C	C C	A G
Pt23	F	- ^a	c.1560+1G > T (IVS8+1G > T)	c.2006T > A (p.V669E)	A A	A A	G G	17 17	A A	A A	T T	G G	G G	G G	G G
Pt24	F	6 months	c.1560+1G > T (IVS8+1G > T)	c.2179C > T (p.R727X)	A A	A A	G G	17 17	A A	A A	T T	G G	G G	G G	G G
Pt25	M	9 months	c.1560+1G > T (IVS8+1G > T)	c.2179C > T (p.R727X)	A A	A A	G G	ND	A A	A A	T T	G G	G G	G G	G G
Pt26	F	Neonate	c.1962delT (p.P654fsX17)	c.1962delT (p.P654fsX17)	G G	A A	G G	16 16	G G	A A	A A	A A	C C	C C	A A
Pt27	M	4 months	c.2179C > T (p.R727X)	c.2179C > T (p.R727X)	A A	A A	G G	17 17	A A	A A	T T	G G	G G	G G	G G
Pt28	F	7 months	c.2179C > T (p.R727X)	c.689_690delCA (p.T231fsX13)	A A	A A	G A	17 16	A G	A C	T A	G A	G C	G C	G G
Pt29	M	11 months	c.2179C > T (p.R727X)	c.2080C > T (p.R694W)	A G	A A	G G	17 16	A G	A A	T A	G A	G C	G C	G A

ND not done

^a Clinical information was not available

Results and discussion

Twenty-nine *mut* MMA patients were studied for mutation analysis. Sequence analysis identified mutations in 95% (55/58) of the disease alleles (Table 2), with 17 mutations being identified in total. Three of the patients (16, 21, and 22) had only one mutation as a heterozygous change each.

Four mutations were novel (p.M1V, c.753_753 + 5delGGTATA, c.1560G > C, and c.2098_2099delAT). The mutation in the translation initiation codon, M1V, has been reported to be pathogenic in other diseases (Lyonnet et al. 1992; Cheadle et al. 1994). The presence of a splice donor site in intron 3 of c.753_753 + 5delGGTATA suggests that this deletion plays a pathogenic role. The sequence flanking this deletion exhibits an intrastrand complementarity (CAAAGGTACTTTG). It is hypothesized that c.753_753 + 5delGGTATA is associated with the formation of the hairpin loop structure in a single-strand DNA (Robinson et al. 1997). A c.1560G > C substitution was identified at the 3' end of exon 8 that

appeared to result in missense-mediated splicing errors. A two-base deletion, c.2098_2099delAT, resulted in a frame-shift and a premature termination.

The total allelic frequency of four mutations (c.385 + 5G > A, p.R369H, p.L494X, and p.R727X) was 55% (32/58). The p.E117X mutation, previously reported as a relatively frequent mutation in Japanese, was found in four alleles (7%) in this study. The mutations p.R93H and p.G648D each appeared more than once in the data from Kobayashi et al. (2006), whereas our data revealed neither. The mutations p.G717V (common mutation in black) and p.N219Y (common mutation in Caucasians) were not found, and p.R108C (common mutation in Hiapanic) was detected in only one allele in this study. Figure 1 summarizes the mutations found in 46 Japanese patients found in 46 Japanese patients (Ogasawara et al. 1994a, b; Toyo-Oka et al. 1995; Mikami et al. 1999; Kobayashi et al. 2006). Worgen et al. (2006) identified exons 2, 3, 6, and 11 as mutation clusters, whereas our data on Japanese patients indicated that 76% (70/92) of the mutations were located in exons (and flanking introns) 2, 6, 8, and 13.

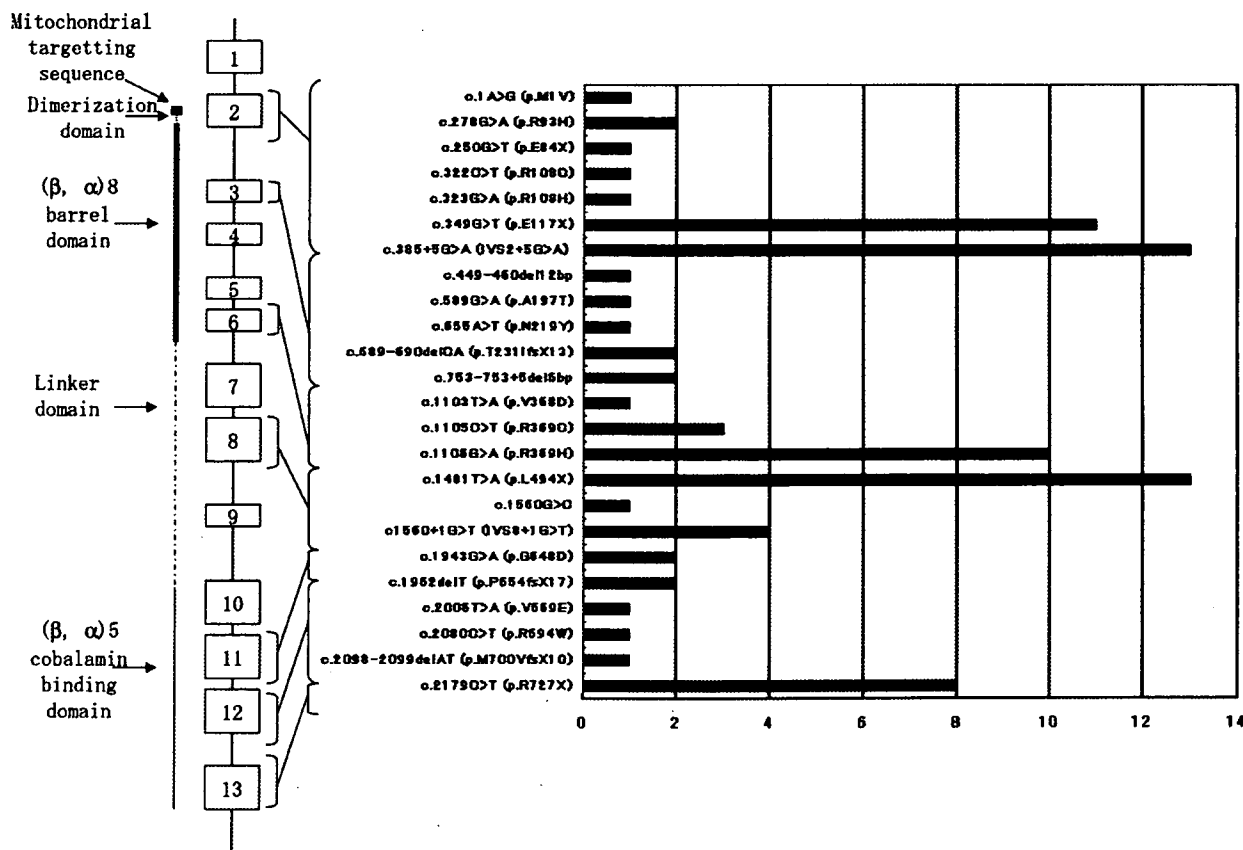


Fig. 1 The distribution of mutations found in 46 Japanese *mut* MMA patients (Ogasawara et al. 1994a, b; Toyo-Oka et al. 1995; Mikami et al. 1999; Kobayashi et al. 2006; and this study)

Table 3 Haplotypes and linked mutations

	c.636 A > G rs2229384	c.1084-50 A > G	c.1495 G > A rs17851388	c.1560+16 T _n	c.1595 G > A rs94735558	c.1676+77 A > C rs9381786	c.1677-164 A > T rs9463483	c.1677-53 A > G rs9473557	c.1808+66 C > G rs9473555	c.2011 G > A rs6458687
Haplotype 1	G	G	G	16	G	A	A	A	C	A
c.349G > T (p.E117X)										
c.1481T > A (p.L494X)										
Haplotype 2	G	A	G	16	G	A	A	A	C	A
c.323G > A (p.R108H)										
c.385+5G > A (IVS2+5G > A)										
c.753_753+5del6bp										
c.1106G > A (p.R369H)										
c.1962delT (p.P654fsX17)										
c.2080C > T (p.R694W)										
Haplotype 3	A	A	G	16	G	C	A	A	C	G
c.1A > G (p.M1V)										
c.1105C > T (p.R369C)										
Haplotype 4	A	A	A	16	G	C	A	A	C	G
c.689_690delCA (p.T2311fsX13)										
c.1105C > T (p.R369C)										
Haplotype 5	A	A	G	17	A	A	T	G	G	G
c.1106G > A (p.R369H)										
c1560G > C										
c1560+1G > T (IVS8+1G > T)										
c.2006T > A (p.V669E)										
c.2179C > T (p.R727X)										
Allele frequency among Japanese (by HapMap Project ^a)	A:0.409 G:0.591				G:0.830 A:0.170	A:0.844 C:0.156			C:0.826 G:0.174	G:0.318 A:0.682

^a The International HapMap Consortium (2003), <http://www.hapmap.org/>

With respect to genotype-phenotype correlations, patient 2 was homozygous for the p.E117X and manifested symptoms on the fifth day of life. Ogasawara et al. (1994b) reported a patient homozygous for p.E117X who showed initial symptoms at the age of 9 months. Patients 5 and 6 were homozygous for c.385 + 5G > A, and the onset was neonatal in both cases: patient 5 is still alive (now 7 years old) and patient 6 died after the first attack. Three patients (7, 8, and 9) were compound heterozygotes of c.385 + 5G > A and p.R369H: patient 7 manifested symptoms at 8 months and these have been kept under well control with vitamin B12 treatment; patients 8 and 9 both showed initial symptoms in the neonatal period and did not respond to vitamin B12. In a previous kinetics study, the V_{\max} value of the p.R369H-mutant enzyme was only 1% of that of the wild type (Janata et al. 1997), and Toyo-Oka et al. (1995) reported that reverse transcription (RT)-PCR did not demonstrate the presence of a normally spliced transcript from fibroblasts of a homozygote of c.385 + 5G > A. We have no explanation why patient 7 responds to vitamin B12 treatment. The clinical features seem to correlate not only with genotype, but also with other unknown environmental factors, such as the nutritional state and/or modifier genes.

Ten single nucleotide polymorphisms (SNPs) were found in the sequenced region, and the haplotypes could be separated into five groups based on five SNP patterns (Table 3). Among the more frequent mutations, p.E117X and p.L494X were linked to haplotype 1; c.385 + 5G > A was linked to haplotype 2; p.R727X was linked to haplotype 5. These frequent mutations appeared to have been spread by the founder effect in the Japanese population. Mutation p.R369H, on the other hand, may be of a double origin (haplotypes 2 and 5). The p.R369 codon contains a CpG dinucleotide, and p.R369H has been found in Turkish, Greek, Caucasian, Hispanic (Worgan et al. 2006), and Korean (Jung et al. 2005) populations.

In conclusion, a limited number of mutations accounted for most of the Japanese *mut* MMA patients, which is in contrast to the results of a previous study on Caucasian patients. Hopefully the results reported here will facilitate the DNA diagnosis of *mut* MMA within the Japanese population.

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Genetic Polymorphisms and Haplotype Structures of the Human *CYP2W1* Gene in a Japanese Population

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ABSTRACT:

A novel human cytochrome P450, designated *CYP2W1*, has recently been identified and is found to be present mainly in tumor cells, particularly in colon cancer cells. In the present study, we report the first systematic investigation of polymorphisms in the human *CYP2W1* gene. Based on denaturing high performance liquid chromatography analyses of polymerase chain reaction products, we analyzed nine exons and exon-intron junctions of the gene in DNA samples from 200 Japanese subjects and identified six single nucleotide polymorphisms (SNP). Three of the novel nonsynonymous SNPs were as follows: 173A>C (Glu58Ala) in exon 1 and 5432G>A (Val432Ile) and 5584G>C (Gln482His) in exon 9. Two previously known nonsynonymous SNPs, that is, 2008G>A

(Ala181Thr) in exon 4 and 5601C>T (Pro488Leu) in exon 9, were also found. On haplotype analyses, in addition to the wild-type *CYP2W1**1A (frequency, 0.295) allele, other alleles, namely, *CYP2W1**1B (0.318), *CYP2W1**2 (0.005), *CYP2W1**3 (0.005), *CYP2W1**4 (0.008), *CYP2W1**5 (0.003), and *CYP2W1**6 (0.368), were also characterized. The most common allele, *CYP2W1**6, exhibited the amino acid substitution Pro488Leu. These results were in good agreement with the expected genotype distributions that were calculated using the Hardy-Weinberg equation. The data on variant alleles and comprehensive haplotype structures would be useful for predicting the metabolic phenotypes of *CYP2W1* substrates in the Japanese population.

Cytochromes P450 (P450s) are mono-oxygenases that play an important role in the oxidative metabolism of many therapeutic drugs and endogenous compounds such as fatty acids, vitamins, and steroids. The P450 enzymes are expressed in high levels in the liver, but these enzymes are also found in extrahepatic tissues. Extrahepatic tissues with high P450 expression levels are the respiratory and gastrointestinal tracts that are exposed to foreign compounds entering the body. The extrahepatic P450 enzymes can be important for tissue-specific metabolic activation or the inactivation of xenobiotic compounds.

CYP2W1, an extrahepatic P450, was recently identified. In adult human tissues, *CYP2W1* mRNA was either not expressed or expressed at very low levels (Choudhary et al., 2005; Aung et al., 2006; Karlgren et al., 2006). In human tumor tissues, *CYP2W1* mRNA is expressed in the colon and adrenal gland (Karlgren et al., 2006). In

particular, high *CYP2W1* mRNA and protein expressions were found in HepG2 cells (Karlgren et al., 2006). Several studies on *CYP2W1* have recently investigated the activity of *CYP2W1* heterologously expressed in *Escherichia coli* and mammalian cells. Karlgren et al. (2006) reported that the *CYP2W1* expressed in HEK293 cells catalyzes the oxidative metabolic conversion of arachidonic acid into 8,9-dihydroxyicosatrienoic acid (DHET), 11,12-DHET, and 14,15-DHET at a small but significant rate. Wu et al. (2006) reported that recombinant *CYP2W1* expressed in *E. coli* metabolizes benzphetamine and catalyzes the activation of several procarcinogens, particularly polycyclic hydrocarbon diols. Yoshioka et al. (2006) also showed that *CYP2W1* expressed in *E. coli* catalyzes the oxidation of indoles.

Wide interindividual differences in metabolic capacity have been detected in many CYP enzymes. For *CYP2W1*, several single nucleotide polymorphisms (SNPs) have been reported thus far, but three of them are located only within exons (<http://www.ensembl.org> and <http://www.hapmap.org>). The SNP 166C>T that is located in exon 1 is silent, but both the other 2 SNPs, namely, 2008G>A in exon 4 and 5601C>T in exon 9, give rise to the amino acid exchanges Ala181Thr and Pro488Leu, respectively.

In the present study, we systematically investigated the variants of *CYP2W1* in a population sample that comprised 200 Japanese subjects. To analyze the protein-coding region of all of the 9 exons and find novel genetic variations, we used a denaturing high performance

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On September 18, 2007, the SNPs detected in this study were not present in dbSNP in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP>), GeneSNPs at the Utah Genome Center (<http://www.genome.utah.edu/genesnps>), or the Human CYP Allele Nomenclature Committee database (<http://www.cypalleles.ki.se>).

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ABBREVIATIONS: P450, cytochrome P450; SNP, single nucleotide polymorphism; DHET, 8,9-dihydroxyicosatrienoic acid; DHPLC, denaturing high performance liquid chromatography; PCR, polymerase chain reaction; CI, confidence interval.

TABLE I
Amplification and DHPLC conditions for CYP2W1 SNP analysis of genomic DNA

Exon	Size bp	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Annealing Temperature °C	PCR Cycles	DHPLC Temperature °C
1	264	ggacggggcccaggaggggagtgga	ggcagctgtccaagcggcaagagct	Slowdown ^a 70.0–55.0	63	64.8, 67.5
2	253	cttgtgggtgaggctgccccgggtg	tgccccacacccagtaggccccct	60.0	35	66.5
3	240	ctgggggtgggaacctgggtcacc	ggcagctccaggccccgggaggggc	60.0	35	65.0, 67.5
4	248	cccctccccgggctggcgtgcct	actccaggctcccccccccaag	60.0	35	64.0
5	264	cctggggctgctctcttctccgc	caggacccctacaggcctcaagga	60.0	35	65.5
6	229	acagaccagatcatcccacgagc	ccccggggcagaaggagccgtctc	60.0	30	66.4
7	275	acgagggatggcgtgccaccaag	cctacccagaggagatggaagggg	60.0	35	66.8
8	232	atcttccccggggccccctctctg	gagccctggaggtccgccccacc	60.0	35	65.4
9	278	agcaggcctggtgcagcccactctg	gctgggaggggagtggtcaggagga	60.0	35	66.8

^aSlowdown protocol: The annealing temperature was decreased after cycle 3 by 1.0°C every three cycles beginning at 70°C and decreased to a "slowdown" annealing temperature of 55°C followed by 15 additional cycles with an annealing temperature of 60°C. The PCR was used with a ramp rate at 2.5°C/s and reaching annealing temperature at 1.5°C/s.

liquid chromatography (DHPLC) system. In addition, cloning methods were used to determine these haplotypes of CYP2W1.

Materials and Methods

Subjects and DNA Samples. Venous blood was obtained from 200 unrelated healthy Japanese volunteers, and the patients were admitted to Tohoku University Hospital. Written informed consent was obtained from all blood donors, and the study was approved by the Local Ethics Committee of Tohoku University Hospital and Tohoku Pharmaceutical University. DNA was isolated from K₂EDTA-anticoagulated peripheral blood by using QIAamp DNA Mini Kits (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions.

PCR Amplification. Table I lists the primer pairs that were used to amplify the entire coding sequence and the exon-intron junctions of the CYP2W1 gene. These primers were designed based on the genomic sequence reported in GenBank (accession number NT_007819). Amplicons of exon 1 were generated using the AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA). PCR reactions were performed using the iCycler (Bio-Rad, Hercules, CA). Moreover, the method relied on the combination of the slowdown method (Bachmann et al., 2003) and the addition of betaine (Sigma-Aldrich, St. Louis, MO) for the region with high GC content (>70%). The PCR protocol was as follows: denaturation at 95°C for 5 min followed by 48 cycles of denaturation at 95°C for 30 s, annealing for 30 s, extension at 72°C for 40 s, and, finally, 15 additional cycles with an annealing temperature of 60°C. The amplicons for exons 2 to 9 were generated using the AmpliTaq Gold PCR Master Mix and betaine addition. The PCR protocol was as follows: denaturation at 95°C for 10 min followed by 30 or 35 cycles of denaturation at 95°C for 30 s, annealing for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min. The annealing temperatures and PCR cycles for the screening of CYP2W1 variants are summarized in Table I.

DHPLC Analysis. The PCR products were analyzed using the DHPLC system (WAVE; Transgenomic, Omaha, NE) (Hiratsuka et al., 2004a,b; Ebisawa et al., 2005; Hiratsuka et al., 2005; Hiratsuka et al., 2006; Sasaki et al., 2006; Hanzawa et al., 2007). Unpurified PCR samples (5 µl) were separated on a heated C18 reverse-phase column (DNASep) by using 0.1 M triethylammonium acetate in water and 0.1 M triethylammonium acetate in 25% acetonitrile at a flow rate of 0.9 ml/min. The software provided with the instrument selected the temperature for the heteroduplex separation in the heterozygous CYP2W1 fragment. Table I summarizes the DHPLC running conditions for each amplicon. The linear acetonitrile gradient was adjusted to the retention time of the DNA peak at 4 to 5 min.

Homozygous nucleotide exchanges can occasionally be distinguished because of a slight shift in the elution time compared with the reference. The addition of an approximately equal amount of wild-type DNA to the samples (1:1) before the denaturation step enabled the reliable detection of homozygous alterations. This was performed routinely for all samples to identify homozygous sequence variations. Therefore, all the samples were analyzed as follows: first, the sample alone to detect heterozygotes; then, after mixing each sample with wild-type DNA to detect homozygous variants. The resultant chromatograms were compared with those of the wild-type DNA.

Direct Sequencing. Both strands of samples with variants as determined by

DHPLC were analyzed using a CEQ 8000 automated DNA sequencer (Beckman Coulter, Fullerton, CA). We also sequenced all samples with chromatographic findings that differed from the wild type to establish links between mutations and specific profiles. We sequenced the PCR products by fluorescent dideoxy termination by using the DTCS DNA Sequencing Kit (Beckman Coulter) in accordance with the manufacturer's instructions.

Haplotype Analysis. To determine the linkage among the polymorphisms identified in this study, PCR reactions were used to amplify long fragments that were obtained from individuals who were heterozygous at sites of interest. Long amplicons (5701 base pairs) were generated with LA Taq DNA polymerase (Takara, Kyoto, Japan). The PCR protocol was as follows: denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 68°C for 5 min, and a final extension at 72°C for 7 min. The forward and reverse primers were 5'-ggacggggcccaggagggagtgga-3' and 5'-gctgggagggagtggtcaggagga-3', respectively. The fragment was run on a low-melting gel, gel-purified, and ligated to the pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA). After ligation, the plasmid was transfected into *E. coli* strain TOP10 (Invitrogen), and single colonies (each containing a plasmid with only one of the two alleles) were grown and subjected to plasmid isolation and sequencing using the CEQ 8000 automated DNA sequencer. In cases of samples having both heterozygous 5601C>T and 166C>T, eight samples were cloned and sequenced to ascertain whether they are found together on the same chromosome.

Results

DHPLC and Sequence Analysis. DHPLC analysis of the CYP2W1 gene for the 200 DNA samples obtained from Japanese individuals revealed chromatographic profiles that were distinct from those of the wild type for exons 1, 4, and 9. Direct sequencing analysis of the deviant DNAs detected by DHPLC revealed a total of 6 different polymorphisms, including 3 novel nonsynonymous polymorphisms (Glu58Ala, Val432Ile, and Gln482His), 2 known nonsynonymous polymorphisms (Ala181Thr and Pro488Leu), and 1 silent SNP (Leu561Leu). The locations and frequencies of these polymorphisms are described in Table 2. We did not observe any variants affecting the recognition sequences of the exon-intron splice site or any variants that would create new putative splice sites near the exon-intron boundaries.

Haplotype Analysis. Based on the concomitant occurrence of the SNPs among the individuals studied and the linkage analysis by the cloning method, the different SNPs can be deduced to comprise 7 haplotypes, as described in Fig. 1. A genomic reference sequence of accession number NT_007819 was defined as the wild-type allele CYP2W1*1A. The other alleles were named according to the recommendations of the CYP allele nomenclature committee (<http://www.cypalleles.ki.se>). The allelic variants (CYP2W1*1B–CYP2W1*6) discovered in this study have been submitted to the CYP alleles web page.

Allele and Genotype Frequencies. The frequency of the alleles and genotypes discovered are listed in Fig. 1 and Table 3, respec-

TABLE 2
Location of SNPs and frequencies of the CYP2W1 gene in 200 DNA samples of Japanese individuals

Exon	Location	Genotype	No. of Subjects (n = 200)	Observed Frequency	Predicted Frequency by
				(95% CI)	Hardy-Weinberg's Law
				%	%
1	166 C>T (Leu56Leu)	C/C	92	46.0 (39.1-52.9)	44.9
		C/T	85	42.5 (35.6-49.4)	44.2
		T/T	23	11.5 (7.1-15.9)	10.9
1	173 A>C (Glu58Ala)	A/A	198	99.0 (97.6-100)	99.0
		A/C	2	1.0 (0-2.4)	1.0
		C/C	0	0 (0)	0
4	2008 G>A (Ala181Thr)	G/G	198	99.0 (97.6-100)	99.0
		G/A	2	1.0 (0-2.4)	1.0
		A/A	0	0 (0)	0
9	5432 G>A (Val432Ile)	G/G	196	98.0 (96.1-99.9)	98.0
		G/A	4	2.0 (0.1-3.9)	2.0
		A/A	0	0 (0)	0
9	5584 G>C (Gln482His)	G/G	199	99.5 (98.5-100)	99.5
		G/C	1	0.5 (0-1.5)	0.5
		C/C	0	0 (0)	0
9	5601 C>T (Pro488Leu)	C/C	80	40.0 (33.3-46.7)	40.1
		C/T	93	46.5 (39.7-53.3)	46.4
		T/T	27	13.5 (8.7-18.3)	13.5

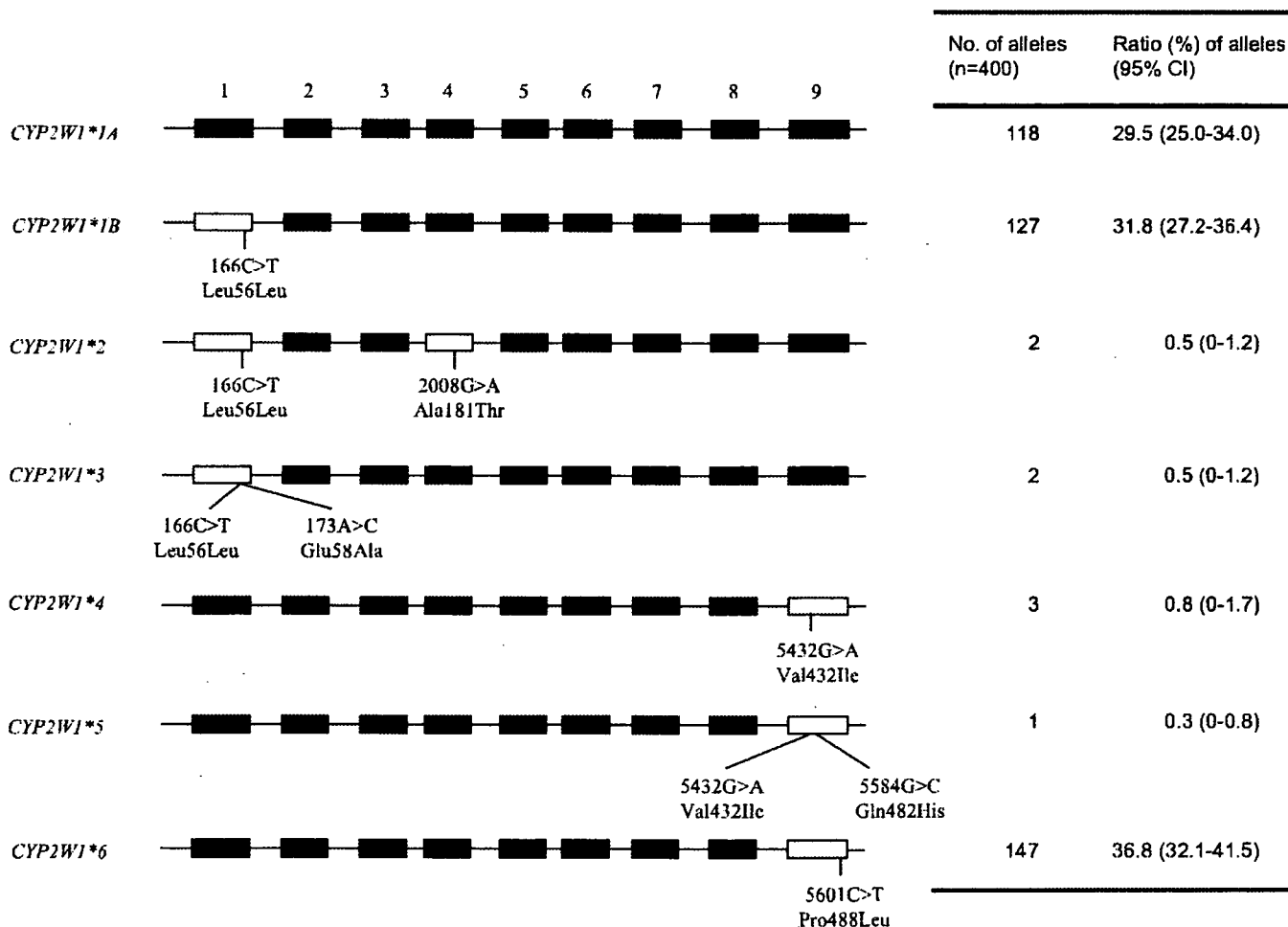


FIG. 1. Allelic variants of the human CYP2W1 gene. Schematic representation of "wild-type" allele for CYP2W1 (CYP2W1*1A) and six variant alleles. The numbering is based on the first nucleotide of the initiation codon that is represented as 1. CYP2W1* alleles were named in accordance with the established nomenclature for the P450 alleles. The frequency of each CYP2W1* allele has been estimated on the population of 200 Japanese volunteers.

tively. CYP2W1*6 was the most common among all the alleles. It occurred at a frequency of 36.8% in our study population (Fig. 1). The frequencies of the various genotypes observed in our Japanese pop-

ulation followed those predicted by the Hardy-Weinberg law (Table 3). These results were in good agreement with the expected genotype distributions that were calculated using the Hardy-Weinberg equation.

TABLE 3
Frequencies of CYP2W1 genotypes in Japanese individuals

CYP2W1 Genotype	No. of Subjects (n = 200)	Observed Frequency	Frequency Predicted
		(95% CI)	by Hardy-Weinberg Law
		%	%
CYP2W1*1A/*1A	21	10.5 (6.2–14.8)	8.7
CYP2W1*1A/*1B	33	16.5 (11.5–21.6)	18.8
CYP2W1*1A/*4	2	1.0 (0–2.4)	0.5
CYP2W1*1A/*5	1	0.5 (0–1.5)	0.2
CYP2W1*1A/*6	40	20.0 (14.5–25.5)	21.7
CYP2W1*1B/*1B	22	11.0 (6.7–15.3)	10.1
CYP2W1*1B/*3	1	0.5 (0–1.5)	0.3
CYP2W1*1B/*6	49	24.5 (18.5–30.5)	23.4
CYP2W1*2/*6	2	1.0 (0–2.4)	0.4
CYP2W1*3/*6	1	0.5 (0–1.5)	0.4
CYP2W1*4/*6	1	0.5 (0–1.5)	0.6
CYP2W1*6/*6	27	13.5 (8.8–18.2)	13.5

Discussion

In the present study, we performed a comprehensive investigation of the genetic variations in CYP2W1 gene. To screen for SNPs in the coding region of this gene, we developed a PCR-DHPLC assay that allows molecular analysis of each exon of the gene. The sequence designated in the National Center for Biotechnology Information as the genome reference sequence with the accession number NT_007819 was defined as the wild-type allele CYP2W1*1A. Five of the six SNPs detected in the CYP2W1 gene in the DNA samples of 200 Japanese subjects resulted in amino acid substitutions. The alleles carrying these alterations were named CYP2W1*2–CYP2W1*6 by the CYP allele nomenclature committee. The variant allele carrying the silent SNP (166C>T, Leu56Leu) was defined as CYP2W1*1B. The most frequent variant allele was CYP2W1*6, followed by CYP2W1*1B and CYP2W1*1A, and their frequencies were observed to be 0.368, 0.318, and 0.295, respectively. Three novel nonsynonymous SNPs, 173A>C (Glu58Ala), 5432G>A (Val432Ile), and 5584G>C (Gln482His), identified in this study were rare, with allele frequencies <1%.

Homology modeling of the human CYP2 family enzymes based on the CYP102 crystal structure (Lewis, 1998) lead to speculation that Val432Ile is located at the heme-binding region. The important region called "P450 signature motif region" comprises the 10-residue P450 signature motif that contains the invariant cysteine that forms the proximal heme ligand. This region and the following α -helix represent one of the most highly conserved sections. This homologous segment is associated with the binding of both heme and redox partners with the common motif Phe-X-X-Gly-X-Arg-X-Cys-X-Gly. In wild-type CYP2W1*1A, this motif is Phe-Ser-Ala-Gly-Arg-Arg-Val-Cys-Val-Gly; in CYP2W1*4 and CYP2W1*5, including Val432Ile, the first valine is changed to isoleucine (Phe-Ser-Ala-Gly-Arg-Arg-Ile-Cys-Val-Gly). For example, the Pro428Thr substitution of CYP2B6 (CYP2B6*2I) in the same region has been clearly identified as being responsible for the decreased protein stability (Klein et al., 2005). Therefore, the homologous segment of the CYP2 family, including CYP2W1, might be closely associated with enzyme activity. Further

studies, such as the use of the recombinant CYP2W1 protein, are required to confirm the function of these variant enzymes.

Karlgren et al. (2006) have reported that CYP2W1 mRNA was detected by real-time PCR of human tumor cells; the highest expression was in colon tumors, but moderate expression was also observed in several adrenal gland tumor cells. They also suggest that CYP2W1 is potentially an important drug target or a useful molecular marker for cancer therapy and diagnosis (Karlgren and Ingelman-Sundberg, 2007). If this is the case, further understanding of the nature of CYP2W1 would be important for cancer therapy.

In summary, this comprehensive investigation of the polymorphisms in the coding region of the CYP2W1 gene identified 6 variant CYP2W1 alleles (CYP2W1*1A–CYP2W1*6). In vitro analysis of recombinant mutated cDNAs as well as phenotyping studies will help in determining the functions of the identified variants.

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